SELF-REPLICATING RNA MOLECULE FROM HEPATITIS C VIRUS

RELATED APPLICATIONS

This application is a divisional of U.S. Application Serial No. 10/309,561, filed December 4, 2004 which is a divisional of U.S. Application Serial No. 10/029,907, filed December 21, 2001, both of which claim, as does the present application priority to U.S. Provisional Application Serial No. 60/257,857 filed on December 22, 2000, the disclosures of all of which are incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to a HCV RNA molecule that self-replicates in appropriate cell lines, particularly to a self-replicating HCV RNA construct having an enhanced efficiency of establishing cell culture replication.

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion and community-acquired non-A non-B hepatitis worldwide. It is estimated that over 200 million people worldwide are infected by the virus. A high percentage of carriers become chronically infected and many progress to chronic liver disease, so called chronic hepatitis C. This group is in turn at high risk for serious liver disease such as liver cirrhosis, hepatocellular carcinoma and terminal liver disease leading to death. The mechanism by which HCV establishes viral persistence and causes a high rate of chronic liver disease has not been thoroughly elucidated. It is not known how HCV interacts with and evades the host immune system. In addition, the roles of cellular and humoral immune responses in protection against HCV infection and disease have yet to be established.

Various clinical studies have been conducted with the goal of identifying pharmaceutical compounds capable of effectively treating HCV infection in patients afflicted with chronic hepatitis C. These studies have involved the use of interferon-alpha, alone and in combination with other antiviral agents such as ribavirin. Such studies have shown that a substantial number of the participants do not respond to these therapies, and of those that do respond favorably, a large proportion were found to relapse after termination of treatment. To date there are no broadly effective antiviral compounds for treatment of HCV infection.

HCV is an enveloped positive strand RNA virus in the Flaviviridae family. The single strand HCV RNA genome is of positive polarity and comprises one open reading frame (ORF) of approximately 9600 nucleotides in length, which encodes a linear polyprotein of approx. 3010 amino acids. In infected cells, this polyprotein is cleaved at multiple sites by cellular and viral proteases to produce structural and non-structural (NS) proteins. The structural proteins (C, E1, E2 and E2-p7) comprise polypeptides that constitute the virus particle (Hijikata, M. et al., 1991, Proc. Natl. Acad. Sci. USA. 88, 5547-5551; Grakoui et al., 1993(a), J. Virol. 67, 1385-1395). The non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) encode for enzymes or accessory factors that catalyze and regulate the replication of the HCV RNA genome. Processing of the structural proteins is catalyzed by host cell proteases (Hijikata, M. et al., 1991, Proc. Natl. Acad. Sci. USA. 88, 5547-5551). The generation of the mature non-structural proteins is catalyzed by two virally encoded proteases. The first is the NS2/3 zinc-dependent metalloprotease which auto-catalyses the release of the NS3 protein from the polyprotein. The released NS3 contains a N-terminal serine protease domain (Grakoui et al., 1993(b), Proc Natl Acad Sci USA, 90, 10583-7; Hijikata, M. et al., 1993, J. Virol. 67, 4665-4675) and catalyzes the remaining cleavages from the polyprotein. The released NS4A protein has at least two roles. First, forming a stable complex with NS3 protein and assisting in the membrane localization of the NS3/NS4A complex (Kim et al., Arch Virol. 1999, 144, 329-343) and second, acting as a cofactor for NS3 protease activity. This membrane-associated complex, in turn catalyzes the cleavage of the remaining sites on the polyprotein, thus effecting the release of NS4B, NS5A and NS5B (Bartenschlager, R. et al., 1993, J. Virol., 67, 3835-3844; Grakoui et al., 1993(a), J. Virol. 67, 1385-1395; Hijikata, M. et al., 1993, J. Virol. 67, 4665-4675; Love, R.A. et al., 1996, Cell, 87, 331-342; reviewed in Kwong et al., 1998 Antiviral Res., 40, 1-18). The C-terminal segment of the NS3 protein also harbors nucleoside triphosphatase and RNA helicase activity (Kim et al., 1995, Biochem. Biophys. Res. Comm., 215, 160-166.). The function of the protein NS4B is unknown. NS5A, a highly phosphorylated protein, seems to be responsible for the Interferon resistance of various HCV genotypes (Gale Jr. et al. 1997 Virology 230, 217; Reed et al., 1997 J. Virol. 71, 7187. NS5B is an RNA-dependent RNA polymerase (RdRp) that is involved in the replication of HCV.

The open reading frame of the HCV RNA genome is flanked on its 5' end by a non-translated region (NTR) of approx. 340 nucleotides that functions as the internal ribosome entry site (IRES), and on its 3' end by a NTR of approximately 230 nucleotides. Both the 5' and 3' NTRs are important for RNA genome replication. The genomic sequence variance is not evenly distributed over the genome and the 5'NTR and parts of the 3'NTR are the most highly conserved portions. The authentic, highly conserved 3'NTR is the object of US patent 5,874,565 granted to Rice et al.

The cloned and characterized partial and complete sequences of the HCV genome have also been analyzed with regard to appropriate targets for a prospective antiviral therapy. Four viral enzyme activities provide possible targets such as (1) the NS2/3 protease; (2) the NS3/4A protease complex, (3) the NS3 Helicase and (4) the NS5B RNA-dependent RNA polymerase. The NS3/4A protease complex and the NS3 helicase have already been crystallized and their three-dimensional structure determined (Kim et al., 1996, Cell, 87, 343; Yem et al. Protein Science, 7, 837, 1998; Love, R.A. et al., 1996, Cell, 87, 331-342 ; Kim et al., 1998, Structure, 6, 89; Yao et al., 1997 Nature Structural Biology, 4, 463; Cho et al., 1998, J. Biol. Chem., 273, 15045). The NS5B RNA dependent RNA polymerase has also been crystallized to reveal a structure reminiscent of other nucleic acid polymerases (Bressanelli et al. 1999, Proc. Natl. Acad. Sci, USA 96, 13034-13039; Ago et al. 1999, Structure 7, 1417-1426; Lesburg et al. 1999, Nat. Struct. Biol. 6, 937-943).

Even though important targets for the development of a therapy for chronic HCV infection have been defined with these enzymes and even though a worldwide intensive search for suitable inhibitors is ongoing with the aid of rational drug design and HTS, the development of therapy has one major deficiency, namely the lack of cell culture systems or simple animal models, which allow direct and reliable propagation of HCV viruses. The lack of an efficient cell culture system is still the main reason to date that an understanding of HCV replication remains elusive.

Although flavi- and pestivirus self-replicating RNAs have been described and used for the replication in different cell lines with a relatively high yield, similar experiments with HCV have not been successful to date (Khromykh et al., 1997, J. Virol. 71, 1497; Behrens et al., 1998, J. Virol. 72, 2364; Moser et al., 1998 J. Virol. 72, 5318). It is known from different publications that cell lines or primary cell cultures can be

infected with high-titer patient serum containing HCV (Lanford et al. 1994 Virology 202, 606; Shimizu et al. 1993 PNAS, USA 90, 6037-6041; Mizutani et al. 1996 J. Virol. 70, 7219-7223; Ikda, et al. 1998, Virus Res. 56, 157; Fourner et al. 1998, J. Gen. Virol. 79, 2376; Ito et al. 1996, J. Gen. Virol. 77, 1043-1054). However, these virus-infected cell lines or cell cultures do not allow the direct detection of HCV-RNA or HCV antigens.

It is also known from the publications of Yoo et al. 1995 J. Virol., 69, 32-38; and of Dash et al., 1997, Am. J. Pathol., 151, 363-373; that hepatoma cell lines can be transfected with synthetic HCV-RNA obtained through *in vitro* transcription of the cloned HCV genome. In both publications the authors started from the basic idea that the viral HCV genome is a plus-strand RNA functioning directly as mRNA after being transfected into the cell, permitting the synthesis of viral proteins in the course of the translation process, and so new HCV particles could form HCV viruses and their RNA detected through RT-PCR. However the published results of the RT-PCR experiments indicate that the HCV replication in the described HCV transfected hepatoma cells is not particularly efficient and not sufficient to measure the quality of replication, let alone measure the modulations in replication after exposure to potential antiviral drugs. Furthermore it is now known that the highly conserved 3' NTR is essential for the virus replication (Yanagi et al., 1999 Proc. Natl. Acad. Sci. USA, 96, 2291-95). This knowledge strictly contradicts the statements of Yoo et al. J. Virol., 69, 32-38(*supra*) and Dash et al., 1997, Am. J. Pathol., 151, 363-373. (*supra*), who used for their experiments only HCV genomes with shorter 3' NTRs and not the authentic 3' end of the HCV genome.

In WO 98/39031, Rice et al. disclosed authentic HCV genome RNA sequences, in particular containing: a) the highly conserved 5'-terminal sequence "GCCAGCC"; b) the HCV polyprotein coding region; and c) 3'-NTR authentic sequences.

In WO 99/04008, Purcell et al. disclosed an HCV infectious clone that also contained only the highly conserved 5'-terminal sequence "GCCAGC".

Recently Lohman et al. 1999 (Science 285, 110-113) and Bartenschlager, R. et al., 1993, J. Virol., 67, 3835-3844(in CA 2,303,526, laid-open on October 3, 2000) disclosed a HCV cell culture system where the viral RNA (I377/NS2-3') self-

replicates in the transfected cells with such efficiency that the quality of replication can be measured with accuracy and reproducibility. The Lohman and Bartenschlager, R. et al., 1993, J. Virol., 67, 3835-3844 disclosures were the first demonstration of HCV RNA replication in cell culture that was substantiated through direct measurement by Northern blots. This replicon system and sequences disclosed therein highlight once again the conserved 5' sequence "GCCAGC". A similar observation highlighting the conservation of the 5'NTR was made by Blight et al. 2000 (Science 290, 1972-1974) and WO 01/89364 published on Nov. 29, 2001.

In addition to the conservation of the 5' and 3' untranslated regions in cell culture replicating RNAs, three other publications by Lohman et al. 2001, J. Virol. 1437-1449 Krieger et al. 2001 J. Virol. 4614-4624 and Guo et al., (2001) J. Virol. 8516-8523 have recently disclosed distinct adaptive mutants within the HCV non-structural protein coding region. Specific nucleotide changes that alter the amino acids of the HCV non-structural proteins are shown to enhance the efficiency of establishing stable replicating HCV subgenomic replicons in culture cells.

Applicant has now found that, contrary to all previous reports, the highly conserved 5'-NTR can be mutated by adaptation to give rise to a HCV RNA sequence that, in conjunction with mutations in the HCV non-structural region, provides for a greater efficiency of transduction and/or replication.

Applicant has also identified novel adaptive mutations within the HCV non-structural region that improves the efficiency of establishing persistently replicating HCV RNA in cell culture.

One advantage of the present invention is to provide an alternative to these existing systems comprising a HCV RNA molecule that self-replicates. Moreover, the present invention demonstrates that the initiating nucleotide of the plus-strand genome can be either an A as an alternative to the G already disclosed.

A further advantage of the present invention is to provide a unique HCV RNA molecule that transduces and/or replicates with higher efficiency. The Applicant demonstrates the utility of this specific RNA molecule in a cell line and its use in evaluating a specific inhibitor of HCV replication.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention provides a 5'-non translated region of the hepatitis C virus wherein its highly conserved guanine at position 1 is substituted for adenine.

Particularly, the present invention provides a hepatitis C virus polynucleotide comprising adenine at position 1 as numbered according to the I377/NS2-3' construct (Lohmann et al. 1999, Science 285, 110-113, Accession # AJ242651).

Particularly, the invention provides a HCV self-replicating polynucleotide comprising a 5'-terminus consisting of ACCAGC (SEQ ID NO. 8).

In a second embodiment, the present invention is directed to a HCV self-replicating polynucleotide encoding a polyprotein comprising one or more amino acid substitution selected from the group consisting of: R(1135)K; S(1148)G; S(1560)G; K(1691)R; L(1701)F; I(1984)V; T(1993)A; G(2042)C; G(2042)R; S(2404)P; L(2155)P; P(2166)L and M(2992)T.

Particularly, the invention is directed to a HCV self-replicating polynucleotide encoding a polyprotein comprising the any one of the amino acid substitutions as described above, further comprising the amino acid substitution E(1202)G.

More particularly, the invention provides a HCV self-replicating polynucleotide encoding a polyprotein comprising a G2042C or a G2042R mutation.

Most particularly, the invention provides for HCV self-replicating polynucleotide comprising a nucleotide substitution G-->A at position 1, and said polynucleotide encodes a polyprotein further comprising a G2042C or a G2042R mutation.

Particularly, the polynucleotide of the present invention can be in the form of RNA or DNA that can be transcribed to RNA.

In a third embodiment, the invention also provides for an expression vector

comprising a DNA form of the above polynucleotide, operably linked with a promoter.

According to a fourth embodiment, there is provided a host cell transfected with the self-replicating polynucleotide or the vector as described above.

In a fifth embodiment, the present invention provides a RNA replication assay comprising the steps of:

- incubating the host cell as described above in the absence or presence of a potential hepatitis C virus inhibitor;
- isolating the total cellular RNA from the cells;
- analyzing the RNA so as to measure the amount of HCV RNA replicated;
- comparing the levels of HCV RNA in cells in the absence and presence of the inhibitor.

In a sixth embodiment, the invention is directed to a method for testing a compound for inhibiting HCV replication, including the steps of:

a) treating the above described host cell with the compound;

b) evaluating the treated host cell for reduced replication, wherein reduced replication indicates the ability of the compound to inhibit replication.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic view of the bi-cistronic replicon RNA. The sequence deviations between the I377/NS2-3' replicon from Lohman et al., 1999 Science 285: 110-113 and the APGK12 replicon are indicated below the replicon. In place of a G nucleotide at the +1 position in the I377/NS2-3'replicon, the APGK12 contains an additional G resulting in GG at the 5' terminus (the first G being counted as position –1). In the linker region between the neo gene and the EMCV IRES sequence two areas deviate from I377/NS2-3': 14 nucleotides (CGCGCCCAGATGTT) which are not present in I377/NS2/3 are inserted at position 1184 in APGK12; 11 nucleotides (1231-1241) present in I377/NS2-3' are deleted to generate APGK-12. In the NS5B coding region, a T at position 8032 was mutated to C to eliminate a NcoI restriction site.

Figure 2 shows Northern blots of RNA-transfected Huh-7 cell lines. 12 µg of total

cellular RNA or control RNA was separated on 0.5% agarose–formaldehyde gels and transferred to Hybond N+ paper, fixed and (Figure 2A) radioactively probed with HCV specific minus-strand RNA that detects the presence of plus-strand replicon RNA. Lanes 1 and 2: positive controls that contain 10^9 copies of *in vitro* transcribed APGK12 RNA. Lane 3: negative control of total cellular RNA from untransfected Huh-7 cells. Lanes 4 and 5: cellular RNA from B1 and B3 cell lines that have integrated DNA copies of the neomycin phosphotransferase gene. Lane 6: total cellular RNA from a Huh-7 cell line, designated S22.3, that harbors high copy number HCV sub-genomic replicon RNA as highlighted by the arrow. Other cell lines have no detectable replicon RNA. Figure 2B is identical to Figure 2A with the exception that the blot was radioactively probed with HCV specific plus-strand RNA to detect the presence of HCV minus-strand RNA. Lanes 1 and 2 are positive control lanes that contain 10^9 copies of full length HCV minus strand RNA. Lane 6, which contains 12 µg of total cellular RNA from cell line S22.3, harbors detectable minus-strand replicon RNA at the expected size of 8 – 9 kilobases. M represent the migration of non-radioactive molecular size markers on the agarose gel. 28s represents the migration of 28s ribosomal RNA and accounts for the detection of this species in a samples of total cellular RNA.

Figure 3 shows indirect immunofluorescence of a HCV non-structural protein in the S22.3 cell line. Indirect immunofluorescence was performed on cells that were cultured and fixed, permeabilized and exposed to a rabbit polyclonal antibody specific for a segment of the HCV NS4A protein. Secondary goat anti-rabbit antibody conjugated with red-fluor Alexa 594 (Molecular Probes) was used for detection. Top panels shows the results of immunofluorescence (40X objective) and the specific staining of the S22.3 cells. The bottom panels represent the identical field of cells viewed by diffractive interference contrast (DIC) microscopy. The majority of S22.3 (Figure 3A) cells within the field stain positively for HCV NS4A protein that localizes in the cytoplasm, whereas the B1 cells (Figure 3B) that fail to express any HCV proteins, only have background level of staining.

Figure 4 shows Western-blots following SDS-PAGE separation of total proteins extracted from three cell lines: (i) naïve Huh-7 cell line, (ii) neomycin resistant Huh-7 cell line B1, and (iii) the S22.3 cell line. Panels A, B, and C, demonstrate the results of western blots probed with rabbit polyclonal antisera specific for neomycin

phosphotransferase (NPT), HCV NS3, and HCV NS5B, respectively. Visualization was achieved through autoradiographic detection of a chemiluminescent reactive secondary \ goat anti-rabbit antibody. Panel A shows that the S22.3 RNA replicon cell line, expresses the NPT protein at levels higher than control B1 cells and that the naïve Huh-7 cell line does not produce the NPT protein. Panels B and C show that only the S22.3 cell line produces the mature HCV NS3 and NS5B proteins, respectively. M represents molecular weight (in kilodaltons) of pre-stained polypeptide markers.

Figure 5A and 5B identify the nucleotide and amino acid sequences respectively that differ from the APGK12 sequence in the different HCV bi-cistronic replicons. The S22.3 adapted replicon is a first generation replicon selected following the transfection of RNA transcribed from the APGK12 template. R3, R7, R16 are second generation replicons that were selected following the transfection of RNA isolated from the S22.3 first generation replicon cell line. Figure 5A: Nucleotide mutations that were characterized in each of the adapted replicons are indicated adjacent to the respective segment of the replicon (IRES, NS3, NS4A, NS5A, and NS5B). Figure 5B: Amino acid numbers are numbered according to the full length HCV poly-protein with the first amino acid in the second cistron corresponding to amino acid 810 in NS2 of I377/NS2-3' construct.

Figure 6 depicts the colony formation efficiency of four *in vitro* transcribed HCV sub-genomic bi-cistronic replicon RNAs. The APGK12 serves as the reference sequence; highlighted are the initiating nucleotides of the HCV IRES in each of the constructs and the amino acid differences (from the APGK12 reference sequence) in the HCV non-structural region for the two R3-rep. Note that the *in vitro* transcribed APGK-12 RNAs that harbor either a 5'G or 5'A form colonies with the same efficiency (ca. 80 cfu/µg in panels A and B) following selection with 0.25 mg/ml G418. RNA isolated from the second generation R3 cell line was reverse transcribed into DNA and cloned into the pAPGK12 vector backbone to generate the R3-rep, which was sequenced and found to encode additional changes that included the L(2155)P substitution in the NS5A segment of the HCV polyprotein (compare R3-rep sequence with the R3 sequence in tables 2 and 3). Various quantities of *in vitro* transcribed R3-rep-5'A RNA, were transfected into naïve Huh-7 cells to determine a colony formation efficiency of 1.2 X 10^6 cfu/µg of RNA (panel C). Various quantities

of R3-rep-5'G were also transfected resulting in a colony formation efficiency of 2 X 10^6 cfu/µg of RNA (panel D).

Figure 7 displays a typical RT-PCR amplification plot (left panel) and the graphical representation of Ct values versus known HCV RNA quantity in a standard curve (right panel). Each of the plotted curves in the left panel, graph the increment of fluorescence reporter signal (delta-Rn) versus PCR cycle number for a predetermined quantity of HCV replicon RNA. The Ct value is obtained by determining the point at which the fluorescence exceeds an arbitrary value (horizontal line). The right panel demonstrates the linear relationship between starting RNA copy number of the predetermined standards (large black dots) and the Ct value. Smaller dots are the Ct values of RNA samples (containing unknown quantity of HCV replicon RNA) from S22.3 cells treated with various concentrations of a specific inhibitor of HCV replication.

Figure 8 shows the effect of increasing concentration of inhibitor A on HCV RNA replicon levels in Huh7 cells. S22.3 cells were grown in the presence of increasing concentrations of inhibitor A starting at 0.5nM and ranging to 1024nM. The inhibitor dose-response curve is the result of 11 concentrations from serial two-fold dilutions (1:1). One control well, without any inhibitor, was also included during the course of the experiment. The cells were incubated for 4 days in a 5% CO_2 incubator at 37 °C. Total cellular RNA was extracted, quantified by optical density. HCV replicon RNA was evaluated by real time RT-PCR and plotted as genome equivalents/µg total RNA as a function of inhibitor concentration

Definitions

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell culture, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989) Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Labs and Ausubel et al. (1994).

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art

and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission (1972) Biochemistry, 11, 1726-1732.

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

The term "DNA segment or molecule or sequence", is used herein, to refer to molecules comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C). These segments, molecules or sequences can be found in nature or synthetically derived. When read in accordance with the genetic code, these sequences can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. The polypeptide can be encoded by a full-length sequence or any portion of the coding sequence, so long as the functional activity of the protein is retained.

A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific structural function that constitute the viral particles. "Structural proteins" defines the HCV proteins incorporated into the virus particles namely, core "C", E1, E2, and E2-p7.

"Non-structural proteins", defines the HCV proteins that are not comprised in viral particles namely, NS2, NS3, NS4A, NS5A and NS5B.

"Restriction endonuclease or restriction enzyme" is an enzyme that has the capacity to recognize a specific base sequence (usually 4, 5 or 6 base pairs in length) in a DNA molecule, and to cleave the DNA molecule at every place where this sequence appears. An example of such an enzyme is *EcoRI*, which recognizes the base sequence G↓AATTC and cleaves a DNA molecule at this recognition site.

"Restriction fragments" are DNA molecules produced by the digestion of DNA with a restriction endonuclease. Any given genome or DNA segment can be digested by a particular restriction endonuclease into at least two discrete molecules of restriction fragments.

"Agarose gel electrophoresis" is an analytical method for fractionating polynucleotide molecules based on their size. The method is based on the fact that nucleic acid molecules migrate through a gel as through a sieve, whereby the smallest molecule has the greatest mobility and travels the farthest through the gel. The sieving characteristics of the gel retards the largest molecules such that, these have the

least mobility. The fractionated polynucleotides can be visualized by staining the gel using methods well known in the art, nucleic acid hybridization or by tagging the fractionated molecules with a detectable label. All these methods are well known in the art, specific methods can be found in Ausubel et al. (*supra*).

"Oligonucleotide or oligomer" is a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. The exact size of the molecule will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide . An oligonucleotide can be derived synthetically, by cloning or by amplification.

"Sequence amplification" is a method for generating large amounts of a target sequence. In general, one or more amplification primers are annealed to a nucleic acid sequence. Using appropriate enzymes, sequences found adjacent to, or in between the primers are amplified. An amplification method used herein is the polymerase chain reaction (PCR) and can be used in conjunction with the reverse-transcriptase (RT) to produce amplified DNA copies of specific RNA sequences.

"Amplification primer" refers to an oligonucleotide, capable of annealing to a RNA or DNA region adjacent to a target sequence and serving as the initiation primer for DNA synthesis under suitable conditions well known in the art. The synthesized primer extension product is complementary to the target sequence.

The term "domain" or "region" refers to a specific amino acid sequence that defines either a specific function or structure within a protein. As an example herein, is the NS3 protease domain comprised within the HCV non-structural polyprotein.

The terms "plasmid" "vector" or "DNA construct" are commonly known in the art and refer to any genetic element, including, but not limited to, plasmid DNA, phage DNA, viral DNA and the like which can incorporate the oligonucleotide sequences, or sequences of the present invention and serve as DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The terminology "expression vector" defines a vector as described above but designed to enable the expression of an inserted sequence following transformation or transfection into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. Such expression control sequences will vary depending on whether the vector is designed to express the operably linked gene *in vitro* or *in vivo* in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements

such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) or RNA, when such nucleic acid has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting/transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, an example of a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome and is inherited by daughter cells through chromosome replication. A host cell or indicator cell can be transfected with RNA. A cell can be stably transfected with RNA if the RNA replicates and copies of the RNA segregate to daughter cells upon cell division. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA or RNA. Transfection methods are well known in the art (Sambrook et al., 1989, Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Labs; Ausubel et al., 1994, Current Protocols in Molecular Biology, Wiley, New York). If the RNA encodes for a genetic marker that imparts an observable phenotype, such as antibiotic resistance, then the stable transfection of replicating RNA can be monitored by the acquisition of such phenotype by the host cell.

As used herein the term "transduction" refers to the transfer of a genetic marker to host cells by the stable transfection of a replicating RNA.

The nucleotide sequences and polypeptides useful to practice the invention include without being limited thereto, mutants, homologs, subtypes, quasi-species, alleles, and the like. It is understood that generally, the sequences of the present invention encode a polyprotein. It will be clear to a person skilled in the art that the polyprotein of the present invention and any variant, derivative or fragment thereof, is auto-processed to an active protease.

As used herein, the designation "variant " denotes in the context of this invention a sequence whether a nucleic acid or amino acid, a molecule that retains a biological activity (either functional or structural) that is substantially similar to that of the original sequence. This variant may be from the same or different species and may be a natural variant or be prepared synthetically. Such variants include amino acid sequences having substitutions, deletions, or additions of one or more amino acids,

provided the biological activity of the protein is conserved. The same applies to variants of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained.

The term "derivative" is intended to include any of the above described variants when comprising additional chemical moiety not normally a part of these molecules. These chemical moieties can have varying purposes including, improving a molecule's solubility, absorption, biological half life, decreasing toxicity and eliminating or decreasing undesirable side effects. Furthermore, these moieties can be used for the purpose of labeling, binding, or they may be comprised in fusion product(s). Different moieties capable of mediating the above described effects can be found in Remington's The Science and Practice of Pharmacy (1995). Methodologies for coupling such moieties to a molecule are well known in the art.

The term "fragment" refers to any segment of an identified DNA, RNA or amino acid sequence and/or any segment of any of the variants or derivatives described herein above that substantially retains its biological activity (functional or structural) as required by the present invention.

The terms "variant", "derivative", and "fragment" of the present invention refer herein to proteins or nucleic acid molecules which can be isolated/purified, synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art. As exemplified herein below, the nucleotide sequences and polypeptides used in the present invention can be modified, for example by *in vitro* mutagenesis.

As used herein, the term "HCV polyprotein coding region" means the portion of a hepatitis C virus that codes for the polyprotein open reading frame (ORF). This ORF may encode proteins that are the same or different than wild-type HCV proteins. The ORF may also encode only some of the functional protein encoded by wild-type polyprotein coding region. The protein encoded therein may also be from different isolates of HCV, and non-HCV protein may also be encoded therein.

As used herein, the abbreviation "NTR" used in the context of a polynucleotide molecule means a non-translated region. The term "UTR" means untranslated region. Both are used interchangeably.

Preferred embodiments

Particularly, the invention provides a HCV self-replicating polynucleotide molecule comprising a 5'-terminus consisting of ACCAGC (SEQ ID NO.8).

According to the first embodiment of this invention, there is particularly provided a HCV polynucleotide construct comprising:

- a 5'-non translated region (NTR) comprising the sequence ACCAGC at, or proximal to, its 5'-terminus;
- a HCV polyprotein coding region; and
- a 3'-NTR region.

In a second embodiment, the present invention is directed to a HCV self-replicating polynucleotide encoding a polyprotein comprising one or more amino acid substitution selected from the group consisting of: R(1135)K; S(1148)G; S(1560)G; K(1691)R; L(1701)F; I(1984)V; T(1993)A; G(2042)C; G(2042)R; S(2404)P; L(2155)P; P(2166)L and M(2992)T.

Particularly, the invention is directed to a HCV self-replicating polynucleotide encoding a polyprotein comprising the any one of the amino acid substitutions as described above, further comprising the amino acid substitution E(1202)G.

Alternatively, the first embodiment of the present invention is directed to HCV self-replicating polynucleotide molecule comprising a G2042C/R mutation.

According to the second embodiment, the present invention particularly provides a HCV polynucleotide construct comprising:

- a 5'-NTR region comprising the sequence ACCAGC at, or proximal to, its 5'-terminus;
- a HCV polyprotein region coding for a HCV polyprotein comprising a G(2042)C or a G(2042)R mutation; and
- a 3'-NTR region.

Preferably, the polynucleotide construct of the present invention is a DNA or RNA molecule. More preferably, the construct is a RNA molecule. Most preferably, the

construct is a DNA molecule.

More particularly, the first embodiment of this invention is directed to a RNA molecule encoded by the DNA molecule selected from the group consisting of: SEQ ID NO. 2, 4, 5, 6, 7, 24 and 25.

Most particularly, the invention provides a DNA molecule selected from the group consisting of: SEQ ID NO. 2, 4, 5, 6, 7, 24 and 25.

In a third embodiment, the invention also is directed to an expression vector comprising DNA forms of the above polynucleotide, operably linked with a promoter.

Preferably, the promoter is selected from the group consisting of: T3, T7 and SP6.

According to a fourth embodiment, there is provided a host cell transfected with the self-replicating polynucleotide or vector as described above. Particularly, the host cell is a eukaryotic cell line. More particularly, the eukaryotic cell line is a hepatic cell line. Most particularly, the hepatic cell line is Huh-7.

In a fifth embodiment, the present invention provides a RNA replication assay comprising the steps of:

a) incubating the host cell as described above under conditions suitable for RNA replication;

b) isolating the total cellular RNA from the cells; and

c) analyzing the RNA so as to measure the amount of HCV RNA replicated.

Preferably, the analysis of RNA levels in step c) is carried out by amplifying the RNA by real-time RT-PCR analysis using HCV specific primers so as to measure the amount of HCV RNA replicated.

Alternatively in this fifth embodiment, the construct comprises a reporter gene, and the analysis of RNA levels in step c) is carried out by assessing the level of reporter expressed.

According to a preferred aspect of the sixth embodiment, the invention is directed to

a method for testing a compound for inhibiting HCV replication, including the steps of:

a) carrying step a) as described in the above assay, in the presence or absence of the compound;

b) isolating the total cellular RNA from the cells; and

c) analyzing the RNA so as to measure the amount of HCV RNA replicated.

d) comparing the levels of HCV RNA in cells in the absence and presence of the inhibitor,

wherein reduced RNA levels is indicative of the ability of the compound to inhibit replication.

Preferably, the cell line is incubated with the test compound for about 3-4 days at a temperature of about 37°C.

EXAMPLES

EXAMPLE 1

Replicon Constructs (APGK-12; Figure 1)

pET9a-EMCV was obtained by ligating an oligonucleotide linker 5' gaattccagatggcgcgcccagatgttaaccagatccatggcacactctagagtactgtcgac 3' (SEQ ID NO.9) to pET-9a (Novagen) that was cut with *EcoRI* and SalI to form the vector pET-9a-mod. This linker contains the following restriction sites: *EcoRI*, AscI, HpaI, NcoI, XbaI, ScaI, SalI. The EMCV IRES was amplified by PCR from the vector pTM1 with primers 5' cggaatcgttaacagaccacaacggtttccctc 3' (SEQ ID NO.10) and 5' ggcgtacccatggtattatcgtgtttttca 3' (SEQ ID NO.11) and ligated into pET-9a-mod via *EcoRI* and NcoI to form pET-9a-EMCV.

The sequence of HCV NS2 to NS5B followed by the 3'UTR of HCV was obtained from the replicon construct I377/NS2-3' (Lohman et al., 1999 Science 285:110-113; accession number: AJ242651) and synthesized by Operon Technologies Inc. with a T to C change at the NcoI site in NS5B at nucleotide 8032. This sequence was released from an GenOp® vector (Operon Technologies) with NcoI and ScaI and transferred into pET-9a-EMCV to form pET-9a-EMCV-NS2-5B-3'UTR.

pET-9a-HCV-neo was obtained by amplification of the HCV IRES from a HCV cDNA isolated from patient serum with primers
5' gcatatgaattctaatacgactcactataggccagcccccgattg 3' (SEQ ID NO.12) containing a T7 promoter and primer
5' ggcgcgccctttggtttttctttgaggtttaggattcgtgctcat 3' (SEQ ID NO.13) and amplification of the neomycin phosphotransferase gene from the vector pcDNA 3.1 (Invitrogen) with primers
5' aaagggcgcatgattgaacaagatggattgcacgca 3' (SEQ ID NO.14) and 5' gcatatgttaactcagaagaactcgtcaagaaggcgata 3' (SEQ ID NO.15). These two PCR fragments were mixed and amplified with primers
5' gcatatgaattctaatacgactcactataggccagcccccgattg 3' (SEQ ID NO.16) and
5' gcatatgttaactcagaagaactcgtcaagaaggcgata 3' (SEQ ID NO.15), cut with Eco RI and HpaI and transferred into pET-9a-mod to form pet-9a-HCV-neo. The EMCV-NS2-5B-3'UTR was released from pET-9a-EMCV-NS2-5B-3'UTR with HpaI and ScaI and transferred into pet-9a-HCV-neo that was cut with HpaI to form pET-9a-APGK12. This insert was sequenced with specific successive primers using a ABI Prism® BigDye™ Terminator Cycle sequencing kit and analyzed on ABI Prism® 377 DNA Sequencer and is shown in SEQ ID NO 1.

RNA *in vitro* transcription
pET-9a-APGK12 DNA was cut with ScaI for expression of the full-length replicon or with BglII for expression of a truncated negative control RNA. DNA was analyzed on a 1% agarose gel and purified by Phenol/Chloroform extraction. RNA was produced using a T7 Ribomax® kit (Promega) followed by extraction with phenol/chloroform and precipitation with 7.5 M $LiCl_2$. RNA was treated with DNAse I for 15 min to remove the DNA template and further purified with an RNeasy® column (Qiagen). RNA integrity was verified on a denaturing formaldehyde 1% agarose gel.

EXAMPLE 2
Primary transfection of Huh7 cells and selection of replicon cell lines
Human hepatoma Huh7 cells (Health Science Research Resources Bank, Osaka, Japan) were grown in 10% FBS/DMEM. Cells were grown to 70% confluency, trypsinized, washed with phosphate buffered saline (PBS) and adjusted to $1x10^7$ cells/ml of PBS. 800 µl of cells were transferred into 0.4cm cuvettes and mixed with 15 µg of replicon RNA. Cells were electroporated using 960µF, 300 volts for ~18

msec and evenly distributed into two 15 cm tissue culture plates and incubated in a tissue culture incubator for 24 hours. The selection of first and second generation replicon cell lines was with 10% FBS/DMEM medium supplemented with 1mg/ml of G418. Cells were selected for 3-5 weeks until colonies were observed that were isolated and expanded.

Following the G418 selection and propagation of Huh-7 cells transfected with APGK12 (SEQ ID NO. 1) RNA, cells that formed a distinct colony were treated with trypsin and serially passed into larger culture flasks to establish cell lines. Approximately 10 X 10^6 cells were harvested from each cell line. The cells were lysed and the total cellular RNA extracted and purified as outlined in Qiagen RNAeasy® preparatory procedures. Figure 2 shows the analysis of 12 μg of total cellular RNA from various cell lines as analyzed on a Northern blot of a denaturing agarose-formaldehyde gel.

Figure 2A is a Northern blot (radioactively probed with HCV specific minus-strand RNA) that detects the presence of plus-strand replicon RNA. Lanes 1 and 2 are positive controls that contain 10^9 copies of *in vitro* transcribed APGK12 RNA. Lane 2 contains the *in vitro* transcribed RNA mixed with 12 μg of total cellular from naïve Huh-7 cells. Lane 3 is a negative control of total cellular RNA from untreated Huh-7 cells. Lanes 4 and 5 contain cellular RNA from the B1 and B3 G418 resistant cell lines that have DNA integrated copies of the neomycin phosphotransferase gene. Lane 6 contains total cellular RNA from a Huh-7 cell line, designated S22.3, that harbors high copy number of HCV sub-genomic replicon RNA as detected by the positive signal in the 8 kilo-base range. Other cell lines have no detectable replicon RNA. Figure 2B is a Northern blot of a duplicate of the gel presented in 2A with the exception that the blot was radioactively probed with HCV specific plus-strand RNA to detect the presence of HCV minus-strand RNA (lanes 1 and 2 are positive control lanes that contain 10^9 copies of full length genomic HCV minus strand RNA); only lane 6, which contains 12 μg of total cellular RNA from cell line S22.3, harbors detectable minus-strand replicon RNA at the expected size of 8 – 9 kilobases. An quantitative estimation of RNA copy number, based on phosphorimager scanning of the Northern blots, is approximately 6 X10^7 copies of plus-strand/μg of total RNA, and 6 x 10^6 copies of minus strand/ μg of total RNA. The presence of the plus-strand and minus-strand intermediate confirms that the HCV sub-genomic RNA is actively

replicating in the S22.3 cell line.

EXAMPLE 3

S22.3 cell line constitutively expresses HCV non-structural proteins.

HCV non-structural protein expression was examined in the S22.3 cell line. Figure 3 displays the result of indirect immunofluorescence that detects the HCV NS4A protein in the S22.3 cell line and not in the replicon negative B1 cell line (a G418 resistant Huh-7 cell line). Indirect immunofluorescence was performed on cells that were cultured and fixed (with 4% paraformaldehyde) onto Lab-tek chamber slides. Cells were permeabilized with 0.2% Triton X-100 for 10 minutes followed by a 1 hour treatment with 5% milk powder dissolved in phosphate-buffered saline (PBS). A rabbit serum containing polyclonal antibody raised against a peptide spanning the HCV NS4A region was the primary antibody used in detection. Following a 2 hour incubation with the primary antibody, cells were washed with PBS and a secondary goat anti-rabbit antibody conjugated with red-fluor Alexa® 594 (Molecular Probes) was added to cells for 3 hours. Unbound secondary antibody was removed with PBS washes and cells were sealed with a cover slip. Figure 3 (top panels) shows the results of immunofluorescence as detected by a microscope with specific fluorescent filtering; the bottom panels represent the identical field of cells viewed by diffractive interference contrast (DIC) microscopy. The majority of S22.3 (Figure 3A) cells within the field stain positively for HCV NS4A protein that localizes in the cytoplasm, whereas the B1 cells (Figure 3B) that fail to express any HCV proteins, only have background level of staining. A small proportion of S22.3 cells express high levels of intensely stained HCV NS4A.

Expression of the proteins encoded by the bi-cistronic replicon RNA was also examined on Western-blots following SDS-PAGE separation of total proteins extracted from: (i) naïve Huh-7 cell line, (ii) neomycin resistant Huh-7 cell line B1, and (iii) the S22.3 cell line. Figure 4 panels A, B, and C, demonstrate the results of western blots probed with rabbit polyclonal antisera specific for neomycin phosphotransferase (NPT), HCV NS3, and HCV NS5B, respectively. Visualization was achieved through autoradiographic detection of a chemiluminescent reactive secondary HRP-conjugated goat anti-rabbit antibody. Figure 4 panel A shows that the S22.3 RNA replicon cell line, expresses the NPT protein at levels higher than B1

cells (which contain an integrated DNA copy of the npt gene) and that the naïve Huh-7 cell line does not produce the NPT protein. Figure 4 panels B and C show that only the S22.3 cell line produces the mature HCV NS3 and NS5B proteins, respectively. The western blots demonstrate that the S22.3 cell line, which harbors actively replicating HCV sub-genomic replicon RNA, maintains replication of the RNA through the high level expression of the HCV non-structural proteins.

EXAMPLE 4

Sequence determination of adapted replicons

Total RNA was extracted from replicon containing Huh7 cells using a RNeasy Kit (Qiagen). Replicon RNA was reverse transcribed and amplified by PCR using a OneStep RT-PCR kit (Qiagen) and HCV specific primers (as selected from the full-length sequence disclosed in WO 00/66623). Ten distinct RT-PCR products, that covered the entire bi-cistronic replicon in a staggered fashion, were amplified using oligonucleotide primers. The PCR fragments were sequenced directly with ABI Prism® BigDye™ Terminator Cycle PCR Sequencing and analyzed on ABI Prism® 377 DNA Sequencer. To analyze the sequence of the HCV replicon 3' and 5' ends a RNA ligation/RT-PCR procedure described in Kolykhalov et al. 1996 J. of Virology, 7, p. 3363-3371 was followed. The nucleotide sequence of S22.3 is presented as SEQ ID NO. 2.

EXAMPLE 5.

Serial Passage of HCV Replicon RNA

The total cellular RNA from the S22.3 cell line was prepared as described above. HCV Replicon RNA copy number was determined by Taqman® RT-PCR analysis and 20 μg of total S22.3 cellular RNA (containing 1 X 10^9 copies of HCV RNA) was transfected by electroporation into 8 X 10^6 naïve Huh-7 cells. Transfected cells were subsequently cultured in 10 cm tissue culture plates containing DMEM supplemented with 10% fetal calf serum (10% FCS). Media was changed to DMEM (10% FCS) supplemented with 1 mg/ml G418 24 hours after transfection and then changed every three days. Twenty-three visible colonies formed three to four weeks post-transfection and G418 selection. G418 resistant colonies were expanded into second generation cell lines that represent the first cell lines harboring serially

passaged HCV Replicon RNA. Three of these cell lines: R3, R7, and R16 were the subject of further analyses. First, the efficiency of transduction by each of the adapted replicons was determined by electroporation of the total cellular RNA (extracted from the R3, R7 and R16) into naïve Huh-7 cells; following electroporation, the transduction efficiency was determined as described above, by counting the visible G418 resistant colonies that arose following 3 to 5 weeks of G418 selection (Table 1). Second, the sequence of the serially passed adapted replicons was determined from the total cellular RNA that was extracted from each of the R3, R7 and R16 replicon cell lines as described in example 4 (SEQ ID NO. 4, 5, 6). Using the pAPGK12 as a reference sequence (SEQ ID NO. 1), the nucleotide changes that were selected in HCV segment of the adapted replicons are presented in Figure 5A. Some of these nucleotide changes are silent and do not change the encoded amino acid whereas others result in an amino acid substitution. Figure 5B summarizes the amino acid changes encoded by the adapted replicons with the amino acid sequence of pAPGK12 as the reference. It is important to note that the reference sequence APGK-12 (SEQ ID NO.1) contains an extra G at the 5'-terminal (5'-GG) that is not maintained in the replicating RNA of the established cell lines. Also noteworthy is that, in addition to G->A at nucleotide 1, there is also an adapted mutation G->C/R at amino acid 2042 (shown as amino acid 1233 in the sequence listing since a.a. 810 of NS2 is numbered as a.a. 1 in SEQ ID) that can be found in all clones analyzed.

TABLE 1

Transfection of Huh-7 cells

RNA	Copies of Replicon	# Colonies	SEQ ID
5 ng APKG12 replicon in 20μg total Huh-7 RNA	1.2×10^9	0	
15 μg APKG12 replicon RNA	3×10^{12}	1 (S22.3)	1
20μg total: S22.3 cellular RNA	3×10^9	23 (3 clones analyzed)	2
R3 cellular RNA	1×10^9	200	4
R7 cellular RNA	1×10^9	20	5
R16 cellular RNA	3×10^8	100	6
cloned R3rep RNA	2.3×10^8	2000	7

EXAMPLE 6

Construction of APGK12 with 5' G-> A substitution (APGK12-5'A, SEQ ID NO.24)

The pAPGK12 DNA was modified to change the first nucleotide in the sequence to replace the 5'GG with a 5'A. The change in the pAPGK12 was introduced by replacing an *EcoRI*/AgeI portion of the sequence with a PCR-generated *EcoRI*/AgeI fragment that includes the mutation. The oligonucleotides used for the amplification were (SEQ ID. NO. 20): 5'-GTG GAC GAA TTC TAA TAC GAC TCA CTA TAA CCA GCC CCC GAT TGG-3' and (SEQ ID. NO. 21): 5'-GGA ACG CCC GTC GTG GCC AGC CAC GAT-3' and generated a 195 bp DNA fragment that was then digested with *EcoRI* and AgeI. The resulting 178 bp restriction fragment was used to replace the *EcoRI* / AgeI fragment in pAPGK12 to generate the pAPGK12-5'A plasmid.

EXAMPLE 7

cDNA CLONING OF THE R3-REPLICON (R3REP).

The cDNA clone of the R3 replicon was produced by RT-PCR of RNA extracted from the R3 cell line. The following two oligonucleotides were used: (SEQ ID. NO. 22): 5'-GTC GTC TTC TCT GAC ATG GAG AC-3' and (SEQ ID. NO. 23): 5'-GAG TTG CTC AGT GGA TTG ATG GGC AGC-3'. The ~4400nt PCR fragment, starting within the NS2 coding region and extending to the 5'-end of the NS5B coding region,

was cloned into the plasmid pCR3.1 by TA cloning (Invitrogen). The SacII / XhoI portion of this R3 sequence was then used to replace the SacII / XhoI fragment present in the pAPGK12 and the pAPGK12-5'A described above. Consequently, two R3 cDNA sequences were generated: (I) R3-Rep-5'G with an initiating 5'G (SEQ ID NO.7), and R3-Rep-5'A (SEQ ID NO.25) with an initiating 5'A. Sequencing of the R3 rep cDNA identified unique nucleotide changes that differ from the original pAPGK12 sequence (see Figure 5A); some of these changes are silent and do not change the encoded amino acid, whereas others do result in an amino acid change (see Figure 5B). The differences between R3 and the R3-rep reflect the isolation of a unique R3-rep cDNA clone encoding nucleotide changes that were not observed from the sequencing of the total RNA extracted from the R3 cell line.

EXAMPLE 8

Efficiency of colony formation with modified constructs

RNA from pAPGK12, pAPGK12-5'A, pR3-Rep and pR3-Rep-5'A was generated by *in vitro* transcription using the T7 Ribomax® kit (Promega) as described in example 1 above. The reactions containing the pAPGK12-5'A and pR3-Rep-5'A templates were scaled-up 10-fold due to the limitation of commercial RNA polymerase in initiating transcripts with 5'-A. The full length RNAs and control truncated RNA for each clone were introduced into 8 x 10^6 naïve Huh-7 cells by electroporation as described in example 2. Replicon RNA was supplemented with total cellular Huh-7 carrier RNA to achieve a final 15-20µg quantity. The cells were then cultured in DMEM medium supplemented with 10% fetal calf serum and 0.25 mg/ml G418 in two 150 mm plates. The lower concentration of G418 was sufficient to isolate and select replicon containing cell lines as none of the transfectants with the control truncated RNA produced any resistant colonies. In contrast, *in vitro* transcribed APGK-12 RNAs that harbor either a 5'G or 5'A form colonies with the same efficiency (ca. 80 cfu/µg in Figure 6 panels A and B) following selection with G418. Various quantities (ranging from 0.1 ng to 1 µg) of the R3-rep-5'A RNA, were transfected into naïve Huh-7 cells to determine a colony formation efficiency of 1.2 X 10^6 cfu/µg of RNA (Figure 6 panel C depicts transfection with 1 µg of RNA). Various quantities (ranging from 0.1 ng to 1 µg) of R3-rep [5'G] were similarly transfected resulting in a colony formation efficiency of 2 X 10^6 cfu/µg of RNA (Figure 6 panel D depicts colony formation with 1µg of RNA). Note that, shown for the first time, HCV subgenomic replicons replicate as efficiently with a 5' A nucleotide in place of the

5'G. APGK12 with a 5'A or 5'G RNA have similar transduction efficiencies. Similarly, R3-Rep RNAs with either the 5'A or 5'G both display the markedly increased transduction efficiency. Notably, the adaptive mutants within the HCV non-structural segment encoded by the R3-Rep provides for a substantial increase in transduction efficiency as depicted by the dramatic increase in colony forming units per μg of transfected RNA.

EXAMPLE 9

Quantification of HCV Replicon RNA Levels in Cell lines

S22.3 cells, or cell lines harboring other adapted replicons, were seeded in DMEM supplemented with 10% FBS, PenStrep and 1μg/mL Geneticin. At the end of the incubation period the replicon copy number is evaluated by real-time RT-PCR with the ABI Prism 7700 Sequence Detection System. The TAQMAN® EZ RT-PCR kit provides a system for the detection and analysis of HCV RNA (as first demonstrated by Martell et al. 1999 J. Clin. Microbiol. 37: 327-332). Direct detection of the reverse transcription polymerase chain reaction (RT-PCR) product with no downstream processing is accomplished by monitoring the increase in fluorescence of a dye-labeled DNA probe (Figure 6). The nucleotide sequence of both primers (adapted from Ruster, B. Zeuzem, S. and Roth, W.K., 1995. Analytical Biochemistry 224:597-600) and probe (adapted from Hohne, M., Roeske, H. and Schreier, E. 1998, Poster Presentation: P297 at the Fifth International Meeting on Hepatitis C Virus and Related Viruses Molecular Virology and Pathogenesis, Venezia-Lido Italy, June 25-28, 1998) located in the 5'-region of the HCV genome are the following:

HCV Forward primer:

5' ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT 3' (SEQ ID NO.17)

HCV Reverse primer:

5' TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG 3' (SEQ ID NO.18)

HCV Probe:

5' FAM-TGG TCT GCG GAA CGG GTG AGT ACA CC-TAMRA 3' (SEQ ID NO.19)

FAM: Fluorescence reporter dye.

TAMRA: Quencher dye.

Using The TAQMAN® EZ RT-PCR kit, the following reaction was set up:

Component	Volume per sample (µL)	Final Concentration
RNase-Free Water	16	-
5X Taqman EZ Buffer	10	1X
Manganese Acetate 25mM	6	3mM
dATP 10mM	1.5	300µM
dCTP 10mM	1.5	300µM
dGTP 10mM	1.5	300µM
dUTP 20mM	1.5	300µM
HCV Forward Primer 10µM	1	200nM
HCV Reverse Primer 10µM	1	200nM
HCV Probe 5uM	2	200nM
rTth DNA Polymerase 2.5U/µL	2	0.1U/µL
AmpErase UNG 1U/µL	0.5	0.01U/µL
Total Mix	45	-

To this reaction mix, 5µL of total RNA extracted from S22.3 cells diluted at 10ng/µL was added, for a total of 50ng of RNA per reaction. The replicon copy number was evaluated with a standard curve made from known amounts of replicon copies (supplemented with 50ng of wild type Huh-7 RNA) and assayed in an identical reaction mix (Figure 7).

Thermal cycler parameters used for the RT-PCR reaction on the ABI Prism 7700 Sequence Detection System were optimized for HCV detection:

Cycle	Temperature (°C)	Time (Minutes)	Repeat	Reaction
Hold	50	2		Initial Step
Hold	60	30		Reverse Transcription
Hold	95	5		UNG Deactivation
Cycle	95	0:15	2	Melt
	60	1		Anneal/Extend
Cycle	90	0:15	40	Melt
	60	1		Anneal/Extend

Quantification is based on the threshold cycle, where the amplification plot crosses a defined fluorescence threshold. Comparison of the threshold cycles provides a highly sensitive measure of relative template concentration in different samples. Monitoring during early cycles, when PCR fidelity is at its highest, provides precise data for accurate quantification. The relative template concentration can be converted to RNA copy numbers by employing a standard curve of HCV RNA with known copy number (Figure 7).

EXAMPLE 10

A specific HCV NS3 protease anti-viral compound inhibits replication of the HCV replicon in S22.3 cell lines.

In order to determine the effect of a specific HCV NS3 protease anti-viral compound on replicon levels in S22.3 cells, the cells were seeded in 24 Well Cell Culture Cluster at 5 X 10^4 cells per well in 500μL of DMEM complemented with 10% FBS, PenStrep and 1μg/mL Geneticin. Cells were incubated until compound addition in a 5% CO_2 incubator at 37 °C. The dose-response curve of the inhibitor displayed 11 concentrations resulting from serial two-fold dilutions (1:1). The starting concentration of compound A was 100nM. One control well (without any compound) was also included in the course of the experiment. The 24 well plates were incubated for 4 days in a 5% CO_2 incubator at 37 °C. Following a 4 day incubation period, the cells were washed once with PBS and RNA was extracted with the RNeasy® Mini Kit and Qiashredder® from Qiagen. RNA from each well was eluted in 50uL of H_2O. The RNA was quantified by optical density at 260nm on a Cary 1E UV-Visible Spectrophotometer. 50 ng of RNA from each well was used to quantify the HCV replicon RNA copy number as detailed in Example 6. The level of inhibition (% inhibition) of each well containing inhibitor was calculated with the following equation (CN = HCV Replicon copy number):

$$\%\cdot inhibition = \left(\frac{CN\cdot control - CN\cdot well}{CN\cdot control}\right) * 100$$

The calculated % inhibition values were then used to determine IC_{50}, slope factor (n) and maximum inhibition (I_{max}) by the non-linear regression routine NLIN procedure of

SAS using the following equation:

$$\%\cdot inhibition = \frac{I_{\max} \times [inhibitor]^n}{[inhibitor]^n + IC_{50}{}^n}$$

Compound A was tested in the assay at least 4 times. The IC_{50} curves were analyzed individually by the SAS nonlinear regression analysis. Figure 8 shows a typical curve and Table 2 shows the individual and average IC_{50} values of compound A. The average IC_{50} of compound A in the replication assay was 1.1nM.

TABLE 2

IC_{50} of compound A in the S22.3 Cell line Replicon Assay.

Compound	IC_{50} (nM)	Average IC_{50} (nM)
A	1.2	
	1.2	
	1.0	
	0.9	
		1.1 ± 0.2

DISCUSSION

The reproducible and robust ex vivo propagation of hepatitis C virus, to levels required for the accurate testing of potential anti-viral compounds, has not been achieved with any system. As an alternative approach to studying the molecular mechanisms of hepatitis C virus RNA replication, selectable self-replicating bi-cistronic RNAs were developed (Lohman et al., 1999, Science 285, 110-113; Bartenschlager, R. et al., 1993, J. Virol., 67, 3835-3844 CA 2,303,526). Minimally, these replicons encode for some or all of the non-structural proteins and also carry a selectable marker such as the neomycin phosphotransferase. Though intracellular steady-state levels of these sub-genomic replicon RNAs among the selected clones is moderate to high, the frequency of generating G418-resistant colonies upon transfection of the consensus RNA described by Lohman et al. or Bartenschlager, R. et al., 1993, J. Virol., 67, 3835-3844 is very low. Less than 100 colonies are generated when 8 million cells are transfected with 1 µg of *in vitro* transcribed bi-

cistronic replicon RNA. A low efficiency of colony formation was first noted by Lohmann et al (1999 et al, Science 285, 110-113). Since then, Lohmann et al. (2001) J. Virol. 1437-1449, Blight et al. 2000, Science 290, 1972-1974,and Guo et al., (2001) J. Virol. 8516-8523, have isolated sub-genomic RNAs with markedly improved efficiencies in the colony formation assay. Lohmann et al., 1999 Science 285, 110-113originally reported that selection of sub genomic replicons may not involve the selection of adaptive mutants as serially passaged RNA did not demonstrate an improved transfection efficiency. Nevertheless, in an effort to characterize the function and fitness of replicating HCV RNA, we serially passaged the replicon RNA that was isolated from the first selected cell-line. Notably, a significant increase in colony forming efficiency was obtained from this experiment, even though the quantity of replicon RNA was orders of magnitude lower than originally used to transfect the *in vitro* transcribed RNA. Furthermore, a second round serial passage of replicon RNA from this first generation clone into naive Huh-7 cells provided for yet another increase in colony formation efficiency (Table 1).

Our analysis of replicating HCV RNAs identified several adaptive mutations that enhance the efficiency of colony formation by up to 4 orders of magnitude. Adaptive mutations were found in many non-structural proteins, as well as in the 5' non-translated region. The substitution of the 5'-GG doublet for a 5'-A as the inaugurating nucleotide of the HCV 5'-UTR is a variant of the HCV genome that has not been previously described, despite the sequencing of innumerable genotypes and subtypes from across the world. Our original replicon that carried a 5'-GG evolved to variants with either a single 5'-A or 5'-G, both of which showed equal transduction efficiency. We describe here the first report of a HCV genome that can tolerate and stably maintain a 5'A extremity. Moreover, we were successful in re-introducing this defined single nucleotide substitution into our cDNA clone and generate *in vitro* transcribed RNA harboring such an extremity to confirm that a 5'A functions as efficiently as a 5'G.

We have identified adaptive amino acid substitutions in the HCV non-structural proteins NS3, NS4A and NS5A in the R3 replicon, and a substitution in NS5B in the R7 clone (see Figure 5B). These mutations, particularly the combination defined by the R3-rep (SEQ ID NO. 7), when reconstituted into a cDNA clone and transcribed onto a RNA replicon, result in a significantly enhanced transduction efficiency of up

to 20,000 fold from the original wild type APGK12 replicon RNA. However, the steady state levels of intracellular replicon RNA were comparable from each of the different isolated clones. This result suggests that the increase in replication efficiency by the adaptive mutations does not result in higher stable intracellular RNA levels due to higher RNA replication, but rather confers increased permissivity for establishing the replicon in a greater number of Huh7 cells. Such a phenotype may be manifested transiently, through an initial increase of the amount of de novo replication, that is required to surpass a defined threshold to establish persistently replicating RNAs within a population of dividing cells.

Recently three other groups also identified other distinct adaptive mutants. Lohmann et al. (2000) reported enhanced transduction efficiencies of up to 10,000 fold with mutations in NS3, NS4B, NS5A and NS5B. Blight et al. 2000, Science 290:1972-1974 reported an augmentation of transduction efficiencies up to 20,000 fold with a single mutation in NS5A whereas Guo et al., (2001) J. Virol. 8516-8523 reported increases in transduction efficiencies of 5,000-10,000 fold with a deletion of a single amino acid in NS5A. The amino acid substitutions that we describe here have not previously been identified as adaptive mutants that enhance the efficiency of RNA transfection and/or replication. One exception is the mutation of E1202G in NS3 that we found in both the R7 and R16 replicons. This adaptation was previously described by Guo et al., (2001) J. Virol. 8516-8523 and Krieger et al (2001) J. Virol. 4614-4624. All other adaptive mutations, without exception, described herein are unpublished.

The development of selectable subgenomic HCV replicons has provided for potential avenues of exploration on HCV RNA replication, persistence, and pathogenesis in cultured cells. However, the low transduction efficiency with the HCV RNA-containing replicons as originally described (Lohmann et al., 1999 Science 285: 110-113) showed that it was not a practical system for reverse genetics studies. The adaptive mutants described herein overcome the low transduction efficiency. In light of the recent descriptions of adaptive mutants by other groups, we note that adaptation can be achieved by distinct mutations in different HCV NS proteins, although the level of adaptation can vary drastically. The replicons encoding adaptive mutants that are described herein are ideally suited for reverse genetic studies to identify novel HCV targets or host cell targets that may modulate HCV

RNA replication or HCV replicon RNA colony formation. The adapted and highly efficient replicons are suitable tools for characterizing subtle genotypic or phenotypic changes that affect an easily quantifiable transduction efficiency.

Lastly, we have used our adapted HCV sub genomic replicon cell-line to demonstrate the proficient inhibition of HCV RNA replication by a specific small molecule inhibitor of the HCV NS3 protease. This is the first demonstration that an antiviral, designed to specifically inhibit one of the HCV non-structural proteins, inhibits HCV RNA replication in cell culture. Moreover, this compound and our S22.3 cell line validate the proposal that RNA replication is directed by the HCV non-structural proteins NS3 to NS5B. The assay that we have described and validated will be extremely useful in characterizing other inhibitors of HCV non-structural protein function in cell culture in a high throughput fashion.

All references found throughout the present disclosure are herein incorporated by reference.